Changing Orders—Primary and Secondary Membrane Transporters Revised

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Cells are surrounded by membranes, which protect them from a hostile environment and allow the maintenance of an internal compartment with a defined composition. In eukaryotes, one or more membranes surround subcompartments, where biochemical processes can take place under compartmentalized conditions. Countless compounds and signals have to be transmitted across these membranes without compromising their important barrier function. Analysis of the growing number of sequenced genomes has revealed that approximately 30 to 40% of encoded proteins contain at least one transmembrane domain, and approximately 10 to 20% of these proteins are thought to be involved in transport processes.[1] Thus, membrane transporters form the largest functional group of proteins, containing more members than, for example, functional groups encoding proteins involved in regulation, transcription, translation, or energy metabolism (for a database and classification of transport proteins see: http://www.biology.ucsd.edu/~msaier/transport/).

Based on their driving force, three classes of active transporters have been defined (Figure 1). Primary transporters use electrical, light, or chemical energy. Well-known examples are the proton-translocating subunits of the respiratory chain, where electron transfer is coupled to the electrogenic transport of protons or sodium ions, or the photosynthetic reaction centers, where absorption of photons is coupled to translocation of protons. Although these systems are extremely important in biology, most primary transport systems use chemical energy, especially ATP, to transport highly diverse substrates. The ATP-binding cassette (ABC) proteins comprise the largest superfamily of primary transporters.[2] Members of this family drive the import as well as export of a wide spectrum of substrates, covering sugars, amino acids, drugs, lipids, peptides and even large proteins. In contrast, secondary transporters use chemiosmotic energy, such as sodium, proton, or charge gradients created by primary-active transporters, to power substrate transport. Since the driving force (proton or sodium gradient) is normally directed inwards, antiport mechanisms are generally used to export substrates, while symport mechanisms operate for import. The class of secondary transporters contains many different families, but approximately 25% belong to the major facilitator superfamily (MFS).[3] The third class of active transporters comprises the phosphoenolpyruvate/carbohydrate phosphotransferase systems (PTS), which phosphorylate the carbohydrate substrate during transport, thereby maintaining the concentration gradient.[4] The PTS consist of two general phosphocarrier proteins, Enzyme I and HPr, and a transporter, Enzyme II. The transporter has three domains (IIA, IIB, and IIC), which can be separate proteins or be linked in any combination (Figure 1). This class of transporters contains relatively few members and is only found in bac teria.

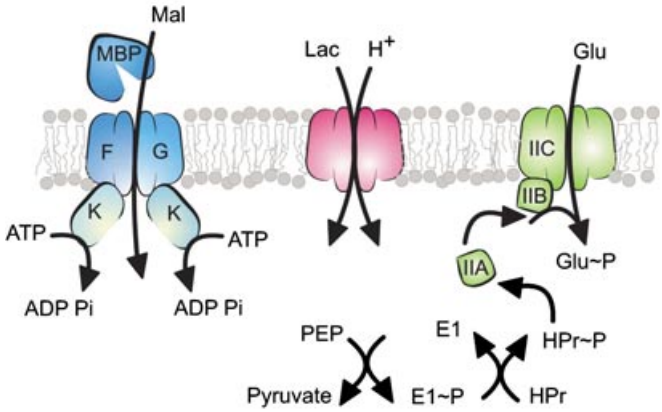

Figure 1. *Examples of the structural organization of primary (the* E. coli *maltose transporter MalFGK, blue), secondary (the* E. coli *lactose transporter LacY, pink), and PTS transporters (the* E. coli *glucose transporter PtsG, green). The* E. coli *maltose transporter consists of a maltose-binding protein (MBP), two transmembrane domains (MalF and MalG), and the nucleotide-binding domain (MalK). The* E. coli *glucose transporter system comprises the general phosphocarrier proteins, HPr and Enzyme I, the cytosolic IIA domain, and the fused IIB and IIC domains.*

After many years of research and in an impressive "tour de force", the crystal structures of several members of the two largest superfamilies of primary (ABC) and secondary transporters (MFS) were solved;[5–9] this marked a new era in membrane biology. The structures showed striking similarities. These similarities were further demonstrated for the homodimeric ABC transporter LmrA of *Lactococcus lactis*, which could be converted from an ATP-dependent transporter to a proton symporter after deletion of the ATP-hydrolyzing domain.[10] This review will focus on what we have learned from similarities and differences in the structures and mechanisms of the two largest families of primary (ABC) and secondary (MFS) transporters.

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Members of both superfamilies are found in all three kingdoms of life, but eukaryotes have relatively more secondary than primary active transporters. MFS transporters show only low sequence homology, but their hydropathy profiles are remarkably similar. MFS transporters commonly have 12 transmembrane helices (TMs).[3] Proteins with 14 or 24 TMs have also been found, but these proteins could be truncated to 12 TMs without loss of activity. The N- and C-terminal 6 helices, which are grouped in two separate transmembrane domains (TMDs), possess weak sequence homology and are normally separated by a long cytoplasmic loop. Interestingly, ABC transporters also contain two TMDs. A canonical TMD of an ABC transporter possesses six TMs, although some have TMDs with either more or less than six TMs.[2] For example, the transporter associated with antigen processing (TAP) is a heterodimer of TAP1 and TAP2, which contain ten and nine TMs, respectively. Interestingly, TAP can be truncated to a 6+6 TM core without affecting the transport function.[11] The truncated domains are, however, essential for the recruitment of tapasin, an accessory protein involved in assembly of a macromolecular peptide-loading complex. Possibly, in other, but certainly not all, ABC transporters containing more than 12 TMs, the transport activity is also located in a 6+6 TM core. ABC proteins also have two conserved nucleotide-binding domains (NBDs) and a binding protein in the case of bacterial importers. The binding protein collects the substrate and delivers it to the transporter, where the NBDs energize its transport by ATP hydrolysis.[12] ABC transporters that function as importers are often composed of an additional fifth protein, while many, especially eukaryotic exporters are assembled by one or two polypeptides.

The NBDs contain several highly conserved motifs, the Walker A and Walker B motifs, which are characteristic of ATP-binding proteins, and the C-loop motif (LSGGQ), unique to ABC proteins, which is also known as the ABC signature motif. X-ray structures of several NBDs have been solved.[13] The NBD forms an L-shaped molecule with two arms, one including the ATP/GTP-binding domain containing the Walker A and B motifs and the other comprising the C-loop. The NBDs are considered to be functional as a dimer. The X-ray structures of the Rad50 dimer,[14] a DNA repair enzyme that shares homology with ABC proteins, a mutated NBD from *M. jannaschii* MJ0796,[15] and *E. coli* MalK are assumed to represent physiologically relevant dimers.[16] In these dimers, the monomers are oriented in a head-to-tail configuration. The two ATP molecules are bound at the interface of the two monomers and sandwiched between the Walker A and B motifs from one monomer and the C-loop of the other monomer.

Crystallization of transport proteins appear to be extremely difficult, but most recently, the structures of two major facilitators, the lactose/proton symporter, LacY[8] and the glycerol-3-phosphate/Pi antiporter, GlpT,[9] both from *E. coli*, have been unraveled (Figure 2). The LacY finally crystallized contained a lactose homologue and the C154G mutation that arrested the protein in the inward facing conformation.[8] GlpT was crystallized as the wild type in the absence of substrate. LacY and GlpT turned out to be very similar and composed of N- and C-terminal TMDs, each with six TMs, symmetrically positioned[17] (Figure 2, lower panel). TM2 and TM5 of the N-terminal domain, and TM7 and TM11 of the C-terminal domain form the dimer interface. Both structures showed a large hydrophilic cavity open to the cytoplasmic side. Moreover, structures of ABC transporters, the lipid A flippase MsbA,[5,6] and the vitamin B12 importer BtuCD[7] have been recently solved (Figure 2). MsbA functions as a dimer. The crystal structures showed an opened (*E. coli*) and more closed (*V. cholera*) conformation with strong differences in the orientation of both NBDs. In the open state, the NBDs are distantly located, while in the closed state they are in direct contact (Figure 1). The orientation of the NBDs in the more closed structure may, however, not represent the physiologically relevant dimer. Remarkably, the TMD dimer interface is formed by the same TMs as in the LacY and GlpT structures, namely TM2 and TM5 of both subunits. The organization of the other helices is, however, different (Figure 2, lower panel). A large cone-shaped cavity open to the cytoplasmic side is observed

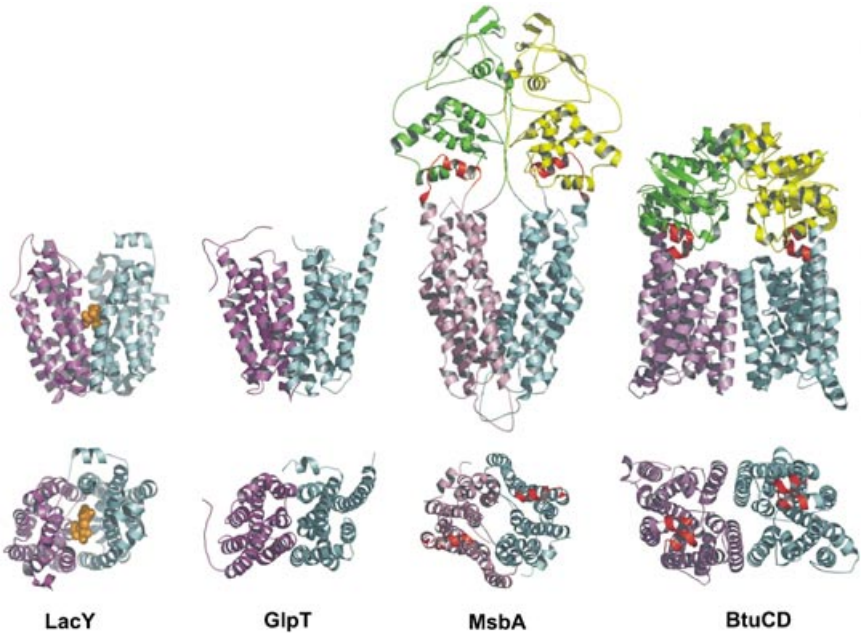

Figure 2. *X-ray structures of* E. coli *LacY,* E. coli *GlpT,* V. cholera *MsbA, and* E. coli *BtuCD. The side view is shown in the upper panel (periplasmic side uppermost), whereas the transmembrane domains are viewed from the cytoplasmic side in the lower panel. The nucleotide-binding domains are shown in yellow and green, the transmembrane domains in cyan and magenta, β-D-galactopyranosyl-1-thio-β-D-galactopyranoside is in orange and the intracellular domain and "L" loop region are in red. The figure is based on the protein databank entries 1PV7, 1PW4, 1PF4, and 1L7V and was generated by using Pymol.*

within the TMDs. The volume of this cavity is strongly reduced in the *V. cholera* structure. However, since in *E. coli* MsbA the buried surface interface between the TMDs is rather small, it seems unlikely that the opened structure represents a physiologically relevant intermediate. In both MsbA structures, the TMDs and NBDs are connected by an α-helical intracellular domain (ICD), which may transfer signals between the NBD and TMD. The vitamin B12 importer, BtuCD, comprises two TMDs (BtuC) and two NBDs (BtuD) (Figure 2). BtuC consists of ten helices instead of six TMs, and their packing is fundamentally different from that observed in MsbA. The interface of the two TMDs is formed by TM5 and TM10, and the TMDs form a cavity at the interface that is open to the periplasmic space. Although the NBDs seem to be slightly separated, they are in the head-to-tail conformation, organized similarly to the sandwiched NBD dimer. Interestingly, the NBDs are in direct contact with a cytoplasmic loop located between TM6 and TM7. This loop folds into two short helices adopting an "L" shape. This "L" loop coincides with the "EAA" motif of many bacterial ABC importers, and has been shown to be involved in communication between the TMD and the NBD.

Only in the LacY structure has a substrate been found. The lactose homologue, β-D-galactopyranosyl-1-thio-β-D-galactopyranoside, was bound at the bottom of the cytosolic cavity between the two TMDs.[8] This binding pocket is in the middle of the membrane (Figure 2, upper panel). Similarly, based on the surface electrostatic potential and the presence of two arginines that are supposed to interact with the phosphate groups of both glycerol-3-phosphate and inorganic phosphate, the binding site of glycerol-3-phosphate in GlpT was predicted to be at the bottom of the cytosolic cavity between the two TMDs.[9] For ABC transporters, it is generally accepted that the substrate-binding site is situated in the TMDs, but the architecture of this site is unknown. Given the size and architecture of MsbA, the cone-shaped cavity between the TMDs facing the cytoplasm was thought to be the substrate-binding site. In BtuCD, the cavity facing the periplasmic space may accommodate vitamin B12. The cavity is, however, less deep than observed in the LacY, GlpT, and MsbA structures. Possibly, this cavity is opened further after interaction with the binding protein or the NBD, as was suggested for the *E. coli* maltose transporter.[16] The vitamin B12-loaded binding protein (BtuF) may dock directly to this putative substrate-binding site.[18] Thus it seems that the substrate-binding site of several members of the MFS and the ABC superfamilies is located between the two pseudosymmetrical TMDs, rather deep in the membrane. This strongly suggests that the translocation pathway is also organized between the two TMDs.

The mechanism of many transporters has long been discussed in terms of an alternating-site model. In this model, a high-affinity binding site facing one side of the membrane is flipped to a low-affinity site on the other side, resulting in transport of the substrate. Evidence for such a mechanism was found for, for example, the multidrug exporter LmrA of *L. lactis*, which exposes a high-affinity substrate-binding site on the inner membrane surface.[19] After vanadate trapping, the high-affinity site on the inner membrane surface was occluded, and a low-affinity site appeared on the outer membrane surface.

The structures of LacY and GlpT support the "alternating access" mechanism of transport. For GlpT, it is speculated that a slight rotation (10°) of the two domains is sufficient to close the pore on the cytosolic site and to open it to the periplasmic site.[9] Based on thiol cross-linking experiments, a larger rotation (~60°) is proposed for LacY.[8] It has been suggested that substrate binding lowers the energy barrier between the two conformations and thus facilitates their conversion. In terms of mechanism, two different models are proposed for LacY and GlpT. In the LacY model (Figure 3A),[8,20] the empty outward-facing conformation is more stable than the inward-facing conformation. Protonation of a glutamate or histidine residue and binding of substrate to the outward-facing conformation decreases the energy barrier enough to trigger a switch to the inward-facing conformation. Proton transfer to a glutamate on the cytosolic side destabilizes substrate binding and leads to release of the substrate and then the proton. The unstable, empty, inward-facing conformation subsequently returns to the outward-facing conformation. For GlpT, the empty inward- and outward-facing conformations are thought to be equally stable.[9] Binding of glycerol-3-phosphate pulls two arginines, located on either side of the dimer interface, together; this lessens the energy barrier enough to allow the switch to the inward-facing conformation. In the inward-facing conformation, glycerol-3-phosphate is released, and replaced by inorganic phosphate because of its higher cytosolic concentration. Phosphate binding also attracts the two arginines and reduces the energy barrier to switch back to the outward-facing conformation. After release of the phosphate on the periplasmic side, glycerol-3-phosphate is again bound because it has a higher affinity (Figure 3B).

It is very likely that ABC transporters also operate via an alternating-access model. How ATP binding/hydrolysis drives the conversion between an inward- and an outward-facing conformation is, however, still under intense debate. Since most isolated ABC transporters show a basal ATPase activity, it was difficult to measure the amount of ATP needed to transport one substrate molecule. Recently, it was demonstrated that two molecules of ATP are hydrolyzed per transported substrate by the glycine–betaine transporter OpuA of *L. lactis*.[21] Several experiments on the human multidrug transporter P-gp/MRP1 have provided evidence that the two ATP-binding sites act in an alternating mode to hydrolyze ATP.[22–24] Hydrolysis of one ATP results in translocation of the substrate whereas hydrolysis of the other ATP has regulatory functions, possibly returning the complex to the starting point of the ATPase cycle (Figure 3C). Remarkably, all structures of NBD dimers are highly symmetric with an equivalence of both ATP-binding sites.[13,25] Thus, other models propose that both ATPs are hydrolyzed in the transport step. In the processive clamp model, binding of the substrate to the cytosolic high-affinity site leads to a conforma-

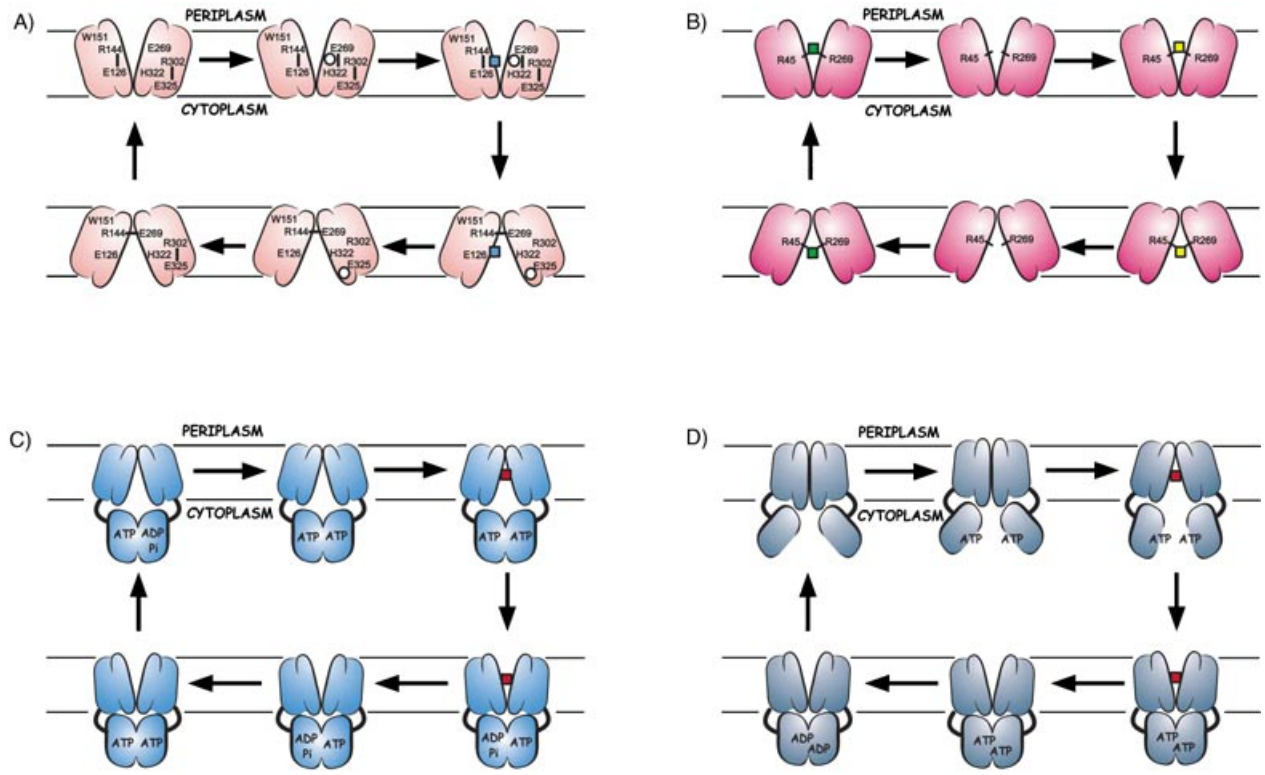

Figure 3. *Models for substrate transport by using alternating access. A) proton/substrate symport as proposed for LacY. B) glycerol-3-phosphate/Pi antiport as proposed for GlpT. C) Alternating site model for substrate export by an ABC transporter. D) Processive clamp model for substrate export by an ABC transporter. Substrates (lactose (blue), glycerol-3-phosphate (green), and Pi (yellow)) are depicted by squares. Protons are depicted as white circles.*

tional change of the TMDs and removes a restraint on the NBD.[15,26] This induces dimer formation and subsequent ATP hydrolysis. Either formation of the dimer or a conformational change induced by ATP hydrolysis results in displacement of the substrate-binding site and transport of the substrate across the membrane, where the substrate is released. Dissociation of the dimer subsequently resets the transporter (Figure 3D). In the case of the maltose transporter of *E. coli*, docking of the substrate-loaded binding protein could trigger formation of the NBD dimer via conformational changes in the TMD.[16] The processive clamp model and the model for maltose transport are mechanistically similar, only the binding protein changes the direction of transport. Whether different ABC transporters have different transport mechanisms, or which of the proposed models of the catalytic cycle is correct remains a subject of intensive research.

As summarized above, primary and secondary transporters of the MFS and ABC superfamilies share several fascinating structural and mechanistic similarities. Could these protein families have evolved from one another? An extensive sequence analysis revealed that integral membrane proteins have probably arisen from small membrane-spanning peptides.[27] These peptides served as building blocks for construction of larger transport proteins through intragenic duplication, triplication, and quadruplication. For example, in the structures of LacY and GlpT, the pseudo-twofold symmetry between the two TMDs and the helix arrangement in two three-helix bundles (TM1/5/6 and TM2/3/4) indicates that the TMDs arose by gene duplication, while the helix bundles evolved by a gene insertion event.[8,9] Protein topology was further shaped by gene fusions, splicing, deletions, insertions, and amino acid substitutions. Apparently, active transport is more easily driven by larger transmembrane proteins that comprise a stable helical bundle than oligomeric assemblies of smaller peptides. Since 6+6, in comparison to 5+5 or 7+7, is statistically a more likely way by which a larger transmembrane protein can be formed by duplication, such a scheme could explain why most, but not all, ABC and MFS transporters consist of a 6+6 dimeric assembly.[27] It has been suggested that primary transporters have evolved from secondary transporters by association with cytosolic energy-coupling subunits (motor domains), thereby acquiring higher transport capacities.[28] An exciting example of such an evolution is the bacterial arsenite transporter ArsAB.[29] Transport of both arsenite and antimonite is driven by hydrolysis of ATP; here ArsA acts as an ATPase that stably associates with the membrane protein ArsB (12 TMs). Transport by the ArsAB complex can only be driven by ATP and is clearly independent of the PMF. It was, however, shown that ArsB alone still provides reduced, but moderate, resistance to arsenite and antimonite. It has been further demonstrated that ArsB extrudes arsenite by a PMF-dependent mechanism and thus works as a secondary transporter. Further indications of such an evolution were recently published

concerning the multidrug ABC transporter LmrA of *L. lactis*.[10] In analogy to ArsAB, the authors show that a truncated version of LmrA, which lacks the NBDs, transported ethidium in symport with a proton and thus functions as a secondary transporter. Although the interpretation of the presented data could still be complicated by the presence of other multidrug transporters in *L. lactis* (J. Lubelski, R. van Merkerk, W. N. Konings, A. J. M. Driessen, unpublished data) and difficulties in controlling the conditions of the ethidium bromide uptake in proteoliposomes, the idea that ABC transporters have evolved from secondary transporters by the acquisition of cytosolic energy-coupling systems and that both use an alternating access mechanism in which the substrate is transported through the middle of the two TMDs, remains very appealing.

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